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A new threat to *Sardina pilchardus* in the northwestern Mediterranean: Genetic validation of the presence of lethal parasites in pelagic eggs and ovaries

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Abstract

Over the past few decades, the fishing industry and species conservationists have grown increasingly concerned about the decrease in catches and poor health of European sardines (*Sardina pilchardus*), particularly in the northwestern Mediterranean region. The species is threatened by a number of factors, including parasite illnesses, rising seawater temperatures, and overfishing. Increasing seawater temperatures due to climate change might lead to the proliferation of protistan parasites, such as *Ichthyodinium chabelardi*. This parasite has been documented in the literature to cause 100% larval mortality in sardines, which may have detrimental effects on fish populations and the fishing industry. We confirmed the existence of *I. chabelardi* in pelagic eggs and the ovaries of European sardines from the Catalan coast (northwestern Mediterranean) by means of stereomicroscopic analysis and a genetic methodology for parasite identification. The incidence of this infection was 22.5% in ovarian tissue and 11.5% in eggs. In this study, we present new data on the vertical transmission of *I. chabelardi* from one generation to another in sardines captured in the Mediterranean region. Interpreting outbreaks of *I. chabelardi*, especially in sardine populations, requires further research into the transmission of infection and improving our understanding of protozoan parasites.

Keywords: *Sardina pilchardus; Ichthyodinium chabelardi*; protistan parasite; vertical transmission; Catalan coast.

Introduction

The cool-temperate pelagic fish *Sardina pilchardus*, also known as the European sardine, plays a crucial ecological role (Cury *et al.,* 2000). According to Ganias *et al*. (2004), this species is a capital breeder because it feeds heavily on plankton during the spring and summer, when primary production is highest, and it stores energy to offset the expense of reproduction during the coldest, most oligotrophic winter months (Albo-Puigserver *et al.,* 2020).

Climate change (Ramírez *et al.,* 2018; Fernández-Corredor *et al.,* 2021), overfishing (Van Beveren *et al.,* 2014; Ramírez *et al.,* 2021), changes in phytoplankton and zooplankton composition (Van Beveren *et al.,* 2014; Albo-Puigserver *et al.,* 2021), pollution, parasite infections (Pennino *et al.,* 2020; Frigola-Tepe *et al.,* 2022; Caballero-Huertas*,* 2023), and the combined effects of these factors (Caballero-Huertas *et al.,* 2023) have all become major threats to European sardine populations in the northwestern Mediterranean Sea. The cumulative impact of these stressors has resulted in unsustainable biomass levels and impaired health status (FAO, 2022). Moreover, a shorter total length and size at first maturity (L50, the length at which 50% of females reach reproductive maturity) have been recorded (Brosset *et al.,* 2016), contributing to reduced fecundity (Ganias*,* 2009). Although sardines spawn in large quantities, only a small percentage of these eggs survive to fertilization and grow into juvenile fish due to their vulnerability to disease, cannibalism, and predators (Garrido *et al.,* 2015; Míguez & Combarro, 2003). Moreover, at this stage, their larvae are very sensitive to changes in temperature (Bernal *et al.,* 2011). Furthermore, Mediterranean sardine recruitment strength is adversely affected by parasitic infections during the planktonic stage. One of the most significant concerns is the infection of fish eggs by the dinoflagellate *Ichthyodinium chabelardi* since this infection has been

observed to result in a 100% mortality rate (Gestal *et al.,* 2006).

Parasitism may affect host physiology, morphology, reproduction, and behaviour (Timi & Poulin, 2020). The inverse relationships between the infection parameters of nematode parasites and the condition of adult sardines from the northwestern Mediterranean have been discussed in several studies (Peninno *et al.,* 2020; Frigola-Tepe *et al.,* 2022; Caballero-Huertas *et al.,* 2023). However, little is known about how parasites can affect sardine eggs and their early development. As primary producers, consumers, and endosymbionts, dinoflagellates play a critical role in marine ecosystems (Fensome et al*.,* 1993). Some behave as parasites, leading to high fish mortality (Rensel & Whyte, 2003)

Ichthyodinium chabelardi has been reported in commercially important species worldwide and in very different ecosystems. In the Mediterranean Sea, it has been found on rare occasions in European sardine and gilthead bream (*Sparus aurata*) (Hollande & Cachon, 1952; Marinaro, 1971, respectively). In the Atlantic Ocean, it has been observed in Atlantic mackerel (*Scomber scombrus*) (Meneses *et al.,* 2003) and European sardine (Gestal *et al.,* 2006; Skovgaard *et al.,* 2009; Carriço *et al.,* 2022). In the Baltic Sea, it has been detected in Atlantic cod (*Gadus morhua*) (Pedersen *et al.,* 1993; Skovgaard *et al.,* 2010) and turbot (*Scopthalmus maximus*) (Pedersen, 1993). Sørensen *et al*. (2014) documented the presence of this parasite in the eggs of wild and domestic European eels (*Anguilla Anguilla*) in both marine and inland lakes (Sørensen *et al.,* 2014). Finally, *I. chabelardi* has been widely identified in artificial tanks containing tropical species such as the leopard coral grouper (*Plectropomus leopardus*) (Mori *et al.,* 2007), yellowfin tuna (*Thunnus albacares*) (Yuasa *et al.,* 2007) and Pacific bluefin tuna (*T. orientalis*) (Ishimaru *et al.,* 2012).

Pathogens use different mechanisms to infect their hosts. There is uncertainty about how the transmission of *I. chabelardi* occurs. If transmission is vertical, as suggested by Pedersen & Køie (1994) in Atlantic cod and turbot, parasites are expected to be present in the gonads. However, if it is horizontal (through the water), as proposed in Yuasa *et al*. (2007), it may involve a different mechanism. *I. chabelardi* (class Syndinophyceae, order Syndiniales) is an endobiotic parasite found in the yolk of embryos and yolk sac larvae of several fish species in both marine ecosystems and aquaculture tanks. While Sørensen *et al*. (2014) proposed the existence of two ribotypes, the genus *Ichthyodinium* is considered a single species. This parasite has a complex life cycle, as shown in Figure 1. *Ichthyodinium chabelardi* multiplies very quickly in the yolk of the egg, and depending on the host species, infections involve two or three successive generations of schizonts. The first generation consists of 1-3 unicellular spheres. The cells undergo several rounds of nuclear division without division of the cytoplasm at the same time, leading to the formation of a large, multinucleated structure. The cytoplasm extends to create tubular projections around each nucleus, and these units each evolve into secondary schizonts. The second generation of schizonts enlarge and undergo division, with daughter cells remaining connected at their ends, potentially forming an extended chain of eight-cell groups (Meneses *et al.,* 2003; Gleason *et al.,* 2019). The last generation adopt a spherical shape and fill the yolk sac after several divisions (Hollande & Cachon, 1953; Meneses *et al.,* 2003; Gleason *et al.,* 2019). This generation of schizonts release zoosporangia into the water (when the egg hatches) but cannot survive swimming freely for a long time; ultimately, in some species, the burst of the yolk sac causes the death of the larvae (Hollande & Cachon, 1953; Meneses *et al.,* 2003; Yuasa *et al.,* 2007).

Thus, previous studies indicate the urgent need for long-term investigations and consider *I. chabelardi* to be an emerging pathogen that could spread into new marine ecosystems and possibly into freshwater ecosystems due to rising seawater temperatures (Sørensen *et al.,* 2014; Gleason *et al.,* 2019). In this context, the primary objective of this study was to use DNA barcoding to develop and validate a genetic method to examine the presence and possible prevalence of *I. chabelardi* in European sardine eggs from the northwestern Mediterranean Sea (Catalan coast). This study also sought to determine whether the infection occurred horizontally (i.e., through the water) or vertically (i.e., through infection of the parental gonads).

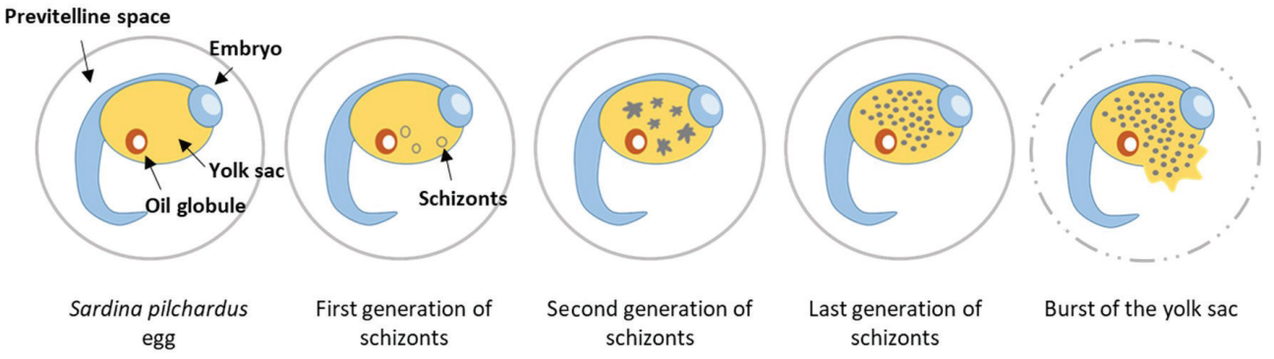

Fig. 1: Life cycle schematic of *Ichhtyiodinium chabelardi* in European sardine eggs. Source: Adapted from Hollande & Chachon, 1953; Meneses *et al.*, 2003; Yuasa *et al*., 2007.

Materials and Methods

Study area and sample collection

The egg samples used in the present study were collected from the coastline of Sant Feliu de Guíxols (Catalan coast, Balearic Sea), which is located in the northwestern Mediterranean Sea (Fig. 2), whereas the sardine ovaries were obtained from three representative locations on the Catalan coast (Palamós, Blanes and Cambrils).

Sample collection of sardine eggs was carried out during the spawning season of European sardines (autumn–winter, average SST 13.73 ± 0.93 °C). Three samplings were performed on December 15, 2020, January 19, 2021, and February 9, 2021. The bongo nets used had a diameter of 50 cm and a mesh size of 300 µm. They were pulled horizontally for 45 minutes/sampling with a boat speed of 1 knot at a depth of 5-15 m. Plankton attached to the nets were preserved in marine water and transported directly to the laboratory.

Visual identification

Plankton in marine water samples were placed into glass plates for stereoscopic evaluation (Zeiss binocular magnifier, Stemi 508). The fish eggs were separated from the sample. Afterwards, the sardine eggs were identified following taxonomic keys and descriptions (Rodriguez *et al.,* 2017) and individually preserved in 96% ethanol in a freezer (-20 °C).

Genetic identification

Species identification of fish eggs

The genetic identification of the fish species was carried out using DNA barcoding techniques. Eggs were individually separated in Eppendorf tubes. Then, the extraction of DNA was performed using the Realpure Spin Genomic DNA Kit from REAL (Kit, n.d.). The extracted DNA was amplified via PCR (*polymerase chain reaction*) using fish barcode primers (COI FIsh-F2: 5′-TCGACTAATCATAAAGATATCGGGAC-3′ and COI Fish-R2: 5′-ACTTCAGGGTGACCGAAGAATCAGAA-3′) (Cutarelli *et al.,* 2014; Keskin & Atar, 2012; 2013). The 25 µl PCR mixture contained 12.5 µl of mix (buffer, MgCl2, dNTPs, Taq), 1 µl of each primer, 8.5 µl of H_2O and 2 µl of extracted DNA. Amplification was performed in an automated thermocycler (Applied Biosystems, 2720 Thermal Cycler) with the following conditions: a denaturing step at 95 °C for 5 min followed by 35 cycles of denaturing at 95 °C for 1 min, annealing at 52 °C for 1 min and 72 °C for 1 min. After the 35 cycles, a final step of 72 to 15 °C over the course of 10 min occurred until the reaction stopped. After PCR, successful amplification was confirmed by agarose gel visualization following electrophoresis. Afterwards, the PCR products were sent to Macrogen, Inc., for cleansing and sequencing using the COI Fish-F2 primer. Sequences were aligned using the software Geneious v7.1.9 (Kearse et al., 2012), and nucleotide identification was established using BLAST (Altschul *et al.,* 1990) for sequence searches in the GenBank database.

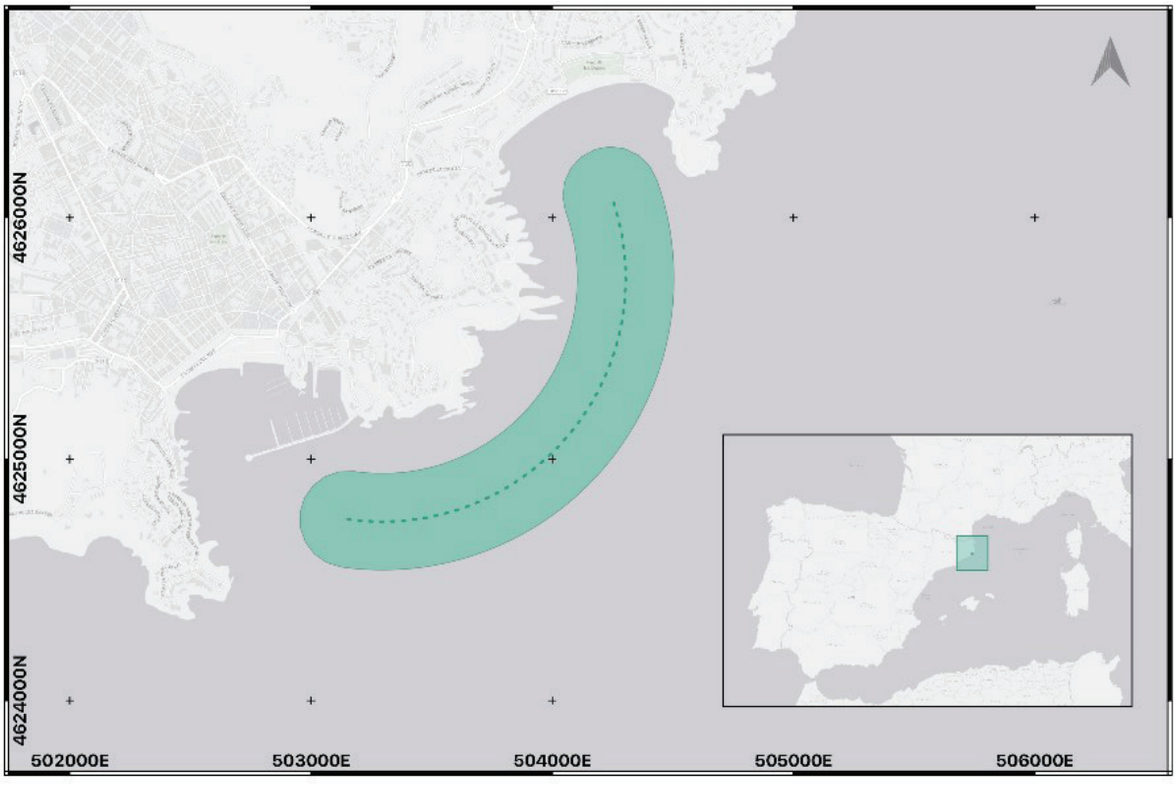

Fig. 2: Map of the sampling route located north of the Catalan coast (in front of Sant Feliu de Guixols).

Parasite identification in eggs

DNA from the eggs identified as sardines was PCR-amplified with primer pairs (Pchab F1: 5′-TCAGCTCTTCGTTGGGGATT-3′ and Pchab R1: 5′-ACAACTTCGGAACAAGGTCC-3′; Pchab F2: 5′-CTGGCACGTTTTCAGCTCTT-3′ and Pchab R2: 5′-ACCAACAACTTCGGAACAAGG-3′) designed in conserved regions of the *Ichthyodinium* nuclear SSU rRNA gene (a small subunit of the ribosome). As a positive control, we used the synthetic sequence of the parasite (G-block) obtained from GenBank under accession number DQ340768.1.

Gradient PCR was carried out at temperatures between 52 °C and 60 °C to determine the optimal annealing temperature. Amplification was performed in an automated thermocycler (Applied Biosystems, 2720 Thermal Cycler) with an annealing temperature of 56 °C. To guarantee the accuracy of our findings, we carefully included three negative controls on each plate. Initially, we used a blank control that was completely DNA-free (nontemplate control). Two DNA samples from sardine muscle tissue, where no parasitic infection is anticipated, were also included. We added two noninfected eggs to each plate to further verify the procedure. Multiple points of reference were provided by this thorough approach, which strengthened the validity of our experimental results. In the second step of parasite verification, nested PCR with specific primers was carried out under the same conditions as those used for the first standard PCR. Two microlitres of the products from standard PCR at a 1/10 concentration served as a template for nested PCR, and the primers used were the *Ichthyodinium*-specific primers NPouF (5'-ACT GTG TGG CAT ACG AAC CA-3') and NPouR (5'-ACA ACT TCG GAA CAA GGT CC-3'), with G-block serving as a positive control.

Finally, the PCR and nested PCR products were subjected to sequencing by Macrogen, Inc., using the NPouF primer. Sequences were aligned using the software Geneious v7.1.9 (Kearse *et al.,* 2012), and their nucleotide similarities were verified via a BLAST (Altschul *et al.,* 1990) search of the GenBank data**b**ase.

Parasite identification in ovaries

Ovaries collected from sardines captured during the 2020–2021 spawning season were preserved in 96% ethanol. Forty gonads were analysed via PCR following the same PCR procedure described previously for *I. chabelardi* identification to determine whether there was female-to-egg transmission. To maximize the detection of the presence of eggs in the gonads, prior to DNA extraction, ovaries were sectioned into three pieces. Three PCR tests were performed for each sample (Fig. 3). PCR amplification was performed at an annealing temperature of 56 °C. Nested PCR amplification of the products of the first PCR at a 1/10 concentration was performed with the NPouF and NPouR primers to improve the reliability of our results. Finally, the samples were subjected to sequencing by Macrogen, Inc., using the primer set NPouF. Sequences were aligned using the software Geneious v7.1.9 (Kearse *et al.,* 2012), and their similarities to other known sequences were established by BLAST searches (Altschul *et al.,* 1990). For the positive control, we PCR-amplified and sequenced the eggs of Atlantic mackerel following a previously described genetic approach to identify *I. chabelardi*. The protozoa in these eggs were previously detected by visual inspection at the Spanish Institute of Oceanography, Oceanographic Center of Vigo (IEO-CSIC) (Spain). A BLAST comparison was performed for the GenBank sequence MH248956.1 (*I. chabelardi* voucher parasite isolated from infected eggs, small subunit ribosomal RNA gene, partial sequence).

Results

Fish egg species identification

A total of 435 European sardine eggs were identified using taxonomic keys and visual inspection (Fig. 4). After genetic validation, 426 eggs were confirmed to belong to the target species. Some samples were discarded because they were not PCR amplified or because the eggs were not genetically identified as sardines.

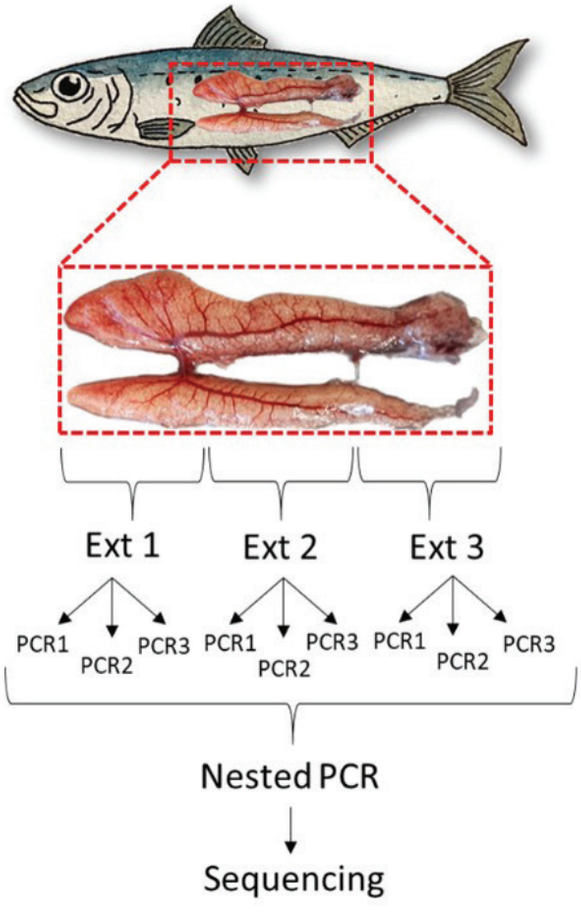

Fig. 3: Schematic of the steps used to detect the presence of *Ichthyodinium chabelardi* in the ovaries of European sardines. Ext refers to DNA extraction.

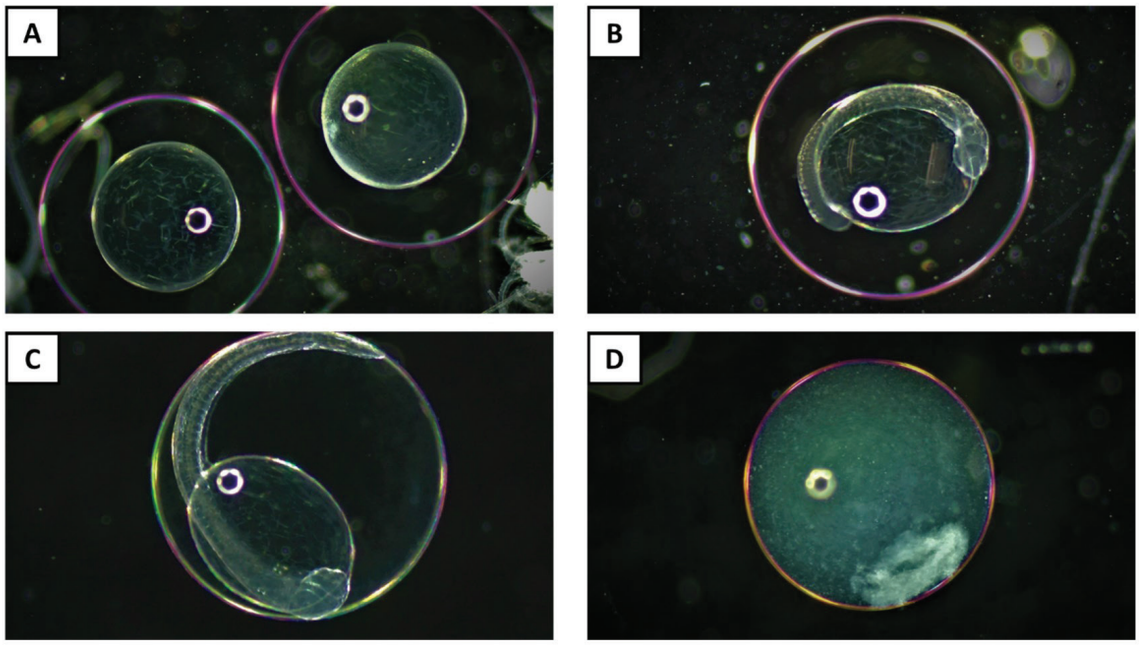

Fig. 4: A. Fertilized sardine eggs in the initial stage visualized under a stereomicroscope; B. Fertilized sardine egg in which a larva can be observed attached to the yolk sac; C. Fertilized sardine egg in which a larva can be observed attached to the yolk sac in a more advanced stage; D. Degraded sardine egg.

Parasite identification

PCR amplification using the Pchab F1/F2 and Pchab R1/R2 primer sets allowed us to identify 34 eggs infected by *I. chabelardi*, for a total of 426 sardine eggs.

Nested PCR, which increases the specificity of PCR (Fig. 5A), confirmed the infection of the eggs by the detection of 15 new positive eggs (49 infected eggs). Therefore, the prevalence of this endoparasite was 11.50 ± 3.03% (95% confidence interval, CI) in European sardine eggs from the northwestern Mediterranean (northern Catalan coast).

Among the 40 ovaries analysed (Fig. 5B), 9 were infected by *I. chabelardi* according to the amplification and sequencing of the subsamples (i.e., extractions of DNA from the gonadal pieces), yielding an infection prevalence of 22.50 ± 12.94% (CI= 95%). In 6 ovaries the parasite was found twice in the same gonad (i.e., in different subsamples or in two different PCRs of the same subsample) (Table 1).

Finally, we successfully tested our protocol on parasite-infected Atlantic mackerel eggs (Fig. 5C), demonstrating the validity of the approach used to assess infection in sardine eggs and gonads. Among the 12 mackerel eggs, 9 were genetically confirmed to be infected by the parasite using our protocol.

A single haplotype was sequenced for all infected samples, including those from eggs and ovaries. The sequence haplotype is available under accession number OR584298 in GenBank. After a BLAST comparison, this haplotype showed the highest similarity with the GenBank sequence MH248956.1 (*I. chabelardi* voucher parasite isolated from infected eggs, small subunit ribosomal RNA gene, partial sequence), with a sequence identity of 99.15% (e-value = 2e-50).

Discussion

This work provides the first genetically verified evidence of *I. chabelardi* infection of European sardines in the Mediterranean region (the Balearic Sea; Catalan coast), including female gonads and planktonic eggs. These intriguing results have two important implications. First, considering that *I. chabelardi* causes a 100% mortality rate in infected eggs of this species, its presence in Mediterranean sardines contributes to the multiple threats this species faces (Gestal *et al.,* 2006). Second, the current investigation offers indications of vertical *I. chabelardi* transmission in European sardines.

Table 1. Summary of results of parasite identification in sardine eggs and ovaries.

Source	N	Method applied	Number infected	Prevalence
Sardine eggs	426	PCR	34	--
		Nested PCR	49	11.50 ± 3.03% (CI= 95%)
Sardine Gonads	40	Nested PCR - triplicates	9	22.50 ± 12.94% (CI= 95%)

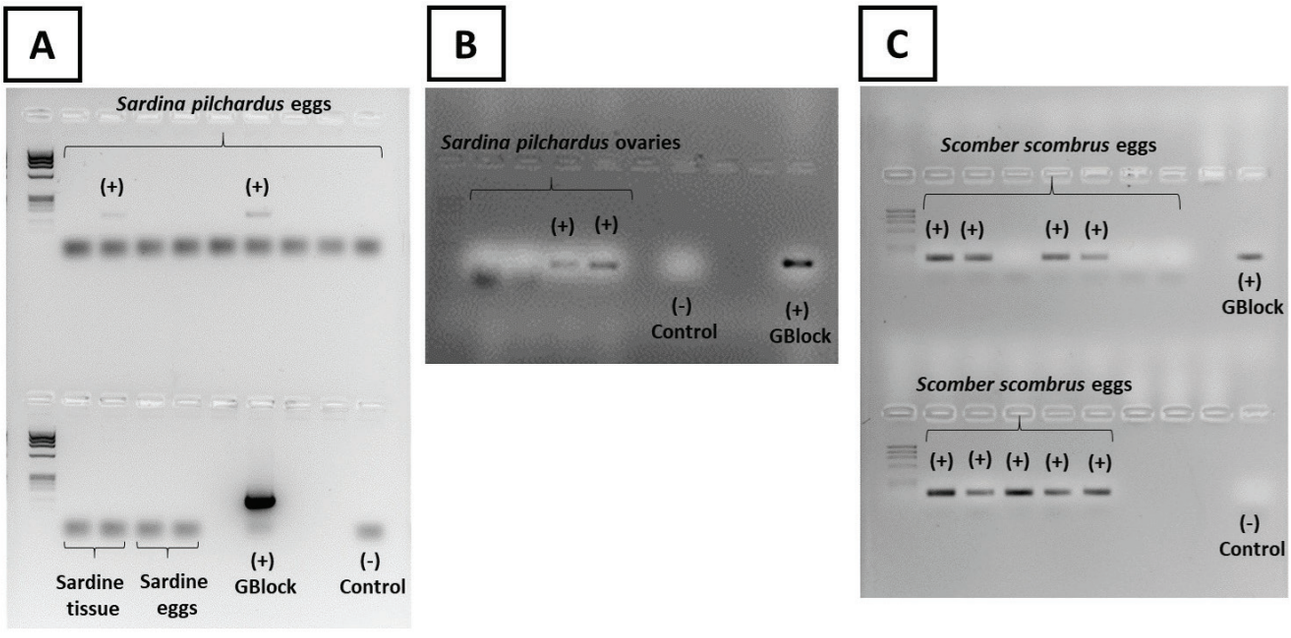

Fig. 5: A. Agarose gel showing infection by *Ichhtyiodinium chabelardi* in two European sardine eggs. G-block was used as a positive control. As negative controls, we added a blank sample with no DNA, two sardine tissue samples and two noninfected eggs onto each plate. B. Agarose gel showing infection by *I. chabelardi* in *Sardina pilchardus* ovaries. G-block was used as a positive control. C. Agarose gel showing infection by *I. chabelardi* in *Scomber scombrus* eggs. G-block was used as a positive control.

As a cosmopolitan parasite, *I. chabelardi* is known to infect various commercially important fish species. Significant variations in infection parameters have been documented across different geographical areas, closely related taxonomic species, seasons, and years (Stratoudakis *et al.,* 2000; Skovgaard *et al.,* 2009). In the Mediterranean Sea, there is little information available regarding the infection of wild fish eggs by *I. chabelardi*. Prevalence has been reported for only European sardines in the Bay of Algier, ranging between 30 and 80% (Hollande & Cachon, 1953). More extensive reports on the infection of European sardine eggs in the Atlantic Ocean are available, with a maximum prevalence ranging from 4.3 to 84.8% (Stratoudakis *et al.,* 2000; Gestal *et al.,* 2006, Carriço *et al.,* 2022). Our study revealed an egg infection prevalence of 11.5% in European sardine eggs on the Catalan coast.

Spatial separation between hosts favours vertical transmission to ensure the propagation of the parasite (Sanders *et al*., 2013), as zoosporangia do not survive freely in the water for a long time (Hollande & Cachon, 1953; Meneses *et al.,* 2003). Horizontal transmission has been reported in yellowfin tuna (*T. albacares)* (Yuasa *et al.,* 2007). In that study, the parasite was found in the water of artificial tanks and not in the gonads. The authors suggested that the parasite took advantage of the micropyle that the sperm uses to enter the egg. Furthermore, in addition to the absence of any signs of penetration on the surface of infected eggs, the present study did not provide any supporting evidence for this hypothesis.

Our results revealed 22.5% infection in the ovaries, and in some ovarian tissues, the parasite was found twice, which confirmed vertical transmission. We hypothesized that *I. chabelardi* could reach females when feeding on infected eggs (from the same or another fish species). Diseases caused by viruses in fish have been found in ovaries and ovarian fluid but not in oocytes (Kocan *et al.,* 2001; Chaves-Pozo *et al.,* 2010). With the methodology that we used, we were unable to determine whether the parasite in the gonad was located inside or outside of the oocyte. It is likely that the parasite migrates into the oocyte from the ovarian tissue, either through the ovarian fluid or directly from the ovarian wall. However, the parasite enters the oocyte at some point during this process.

Although we reported vertical transmission in European sardines, we cannot exclude the possibility that parasite development is also associated with the environment (Yuasa *et al.,* 2007). Sequencing water samples using state-of-the-art environmental DNA methodologies could be key in future studies to identify the presence of *I. chabelardi* DNA, as this DNA may indicate horizontal transmission through the environment. However, detecting the parasite alive can be challenging, as mentioned in the introduction. Zoosporangia do not survive freely in water for a long time (Hollande & Cachon, 1953; Meneses *et al.,* 2003), and traces of parasite DNA can persist for longer periods in the sea (Ruppert *et al.,* 2019). However, this method can be expensive, and negative results may not necessarily imply the absence of the parasite.

Other fish pathogens are transmitted both horizontally and vertically (bacterial, viral, and microsporidian pathogens; e.g., Brown *et al.,* 1997; Sanders *et al.,* 2013; Yamkasem *et al.,* 2019; Caballero-Huertas *et al.,* 2021). The transmission method of an infectious agent may influence parasite virulence. Pathogens that are vertically transmitted tend to exhibit lower virulence due to the additional selection pressure for the survival of the infected female host (Sanders *et al.,* 2013). Therefore, the variations in

virulence observed among different regions and species could also be partly affected by the mode of transmission.

Ichthyodinium chabelardi infection causes the yolk sac of sardine eggs to burst, which could indicate that the prevalence of infection in pelagic sardine eggs is underestimated and that its full extent is not known. The location of our sampling may also have had an impact on the outcome. There may be a small amount of variation in infection rates between sites. According to Yuasa *et al*. (2007), some host species are also more prone to infection, which can affect how parasites develop in eggs and cause embryos or hatched larvae to die. Future research may therefore focus primarily on analysing the intensity of infection or the quantity of parasites present in infected fish. Infected embryos, especially those of European sardines, Atlantic mackerel and yellowfin tuna, exhibit opaque colours due to severe infection. In these species, during the last generation of schizonts, the yolk sac becomes filled with parasite cells, depleting the embryo's energy reserves (Meneses *et al*., 2003) and ultimately leading to the death of the embryo or hatched larva. Given the high prevalence of infection in these species, such as the reported 46% prevalence in Atlantic mackerel eggs (Meneses *et al*., 2003), this could pose a significant concern for the population sustainability of these fish.

The life cycle of *I. chabelardi* is influenced mostly by salinity and water temperature (Skovgaard *et al*., 2010). Fish egg and larval mortality due to infection by *I. chabelardi* has been mostly reported in studies associated with temperate or tropical waters with high salinity (Hollande & Cachon, 1952; Meneses *et al*., 2003; Yuasa *et al*., 2007). Atlantic cod and turbot, which inhabit the colder and less salty Baltic Sea, exhibit lower infection rates. In these species, the eggs do not exhibit opaque colouration and do not undergo yolk sac rupture. Flagellated cells have not been detected in these species, and the parasite lacks chloroplasts and trichocysts (Skovgaard *et al*., 2010). Hence, these species are unsuitable hosts for the parasite, resulting in cell disintegration and the survival of fish embryos. In another example, a study by Sørensen *et al*. (2014) revealed no lethal impact on European eel eggs.

The generalist parasite *I. chabelardi* might be a potential threat to both aquaculture (Mori *et al*., 2007; Yuasa *et al*., 2007) and wild fish populations. The importance of the fish industry in sustaining the growth of the human population makes it imperative to investigate the effects of this infectious agent on marine fish eggs. Furthermore, habitat destruction, the introduction of exotic species, and rising sea temperatures caused by climate change all contribute to the growth of parasites, particularly the increasing prevalence of protozoan parasites such as *I. chabelardi* (Gleason *et al*., 2019). Fish species from northern and colder latitudes, where *I. chabelardi* did not previously cause egg mortality, may now experience an increase in egg mortality due to rising seawater temperatures. Considering the 100% mortality rate caused by this parasite in European sardines, a sudden outbreak (caused by rising seawater temperature) could have devastating consequences for their population. Therefore, it is important to gather more information about the life cycle and transmission methods of this parasite to mitigate its impact on the sardine population and prevent economic losses in the fishing industry.

Conclusion

European sardines from the northwestern Mediterranean Sea are susceptible to parasitic infection during their planktonic stages. This study presents the first genetically validated evidence of *I. chabelardi* infecting European sardines, including both pelagic eggs and ovaries, and provides new data on the vertical transmission of *I. chabelardi* in European sardines. Given that this parasite has been reported to kill 100% of infected eggs (Gestal *et al*., 2006), a long-term investigation could be essential for analysing the intra-annual dynamics of infections across various locations in the Mediterranean region and determining whether the prevalence of this parasite increases or remains stable.

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Conflicts of interest: The authors have no conflicts of interest to declare. **Data availability statement:** The sequence haplotype is available under accession number OR584298 in GenBank. **Author Contributions:** Xènia Frigola-Tepe: conceptualization, sample collection, data analyses, investigation, writing, and visualization; Núria Pérez-Bielsa: investigation; Marta Caballero-Huertas: sample collection, writing, and revision; Judith Ollé-Vilanova: data analyses and revision; Marta Muñoz: conceptualization, revision, supervision, and project administration; Jordi Viñas: conceptualization, revision, supervision, and project administration.

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